Country	:	France
Document No.	:	WO 94/05787
Document Type	:	Patent Application
Language	:	French
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IPC	:	C12N 15/12, 15/63, 15/82 C07K 9/00, A61K 37/02
Application Date	:	September 6, 1993
Publication Date	:	March 17, 1994
Foreign Language Title	:	Glycopeptides, Leur Procede d'obtention et Leurs Applications Biologiques
English Language Title	:	GLYCOPEPTIDES, METHOD OF OBTAINING THEM AND BIOLOGICAL APPLICATIONS THEREOF

/1[1]

This invention relates to glycopeptides, their procurement as well as their biological uses.

More particularly, it relates to glycopeptides that, in particular, offer antibacterial properties.

It is known that antibacterial peptides were isolated from insects. Certain species of insects, as a matter of fact, present effective resistance against bacteria. Recent studies showed that this defense to a great extent is based on the rapid synthesis (2 to 6 hours) of several families of peptides or polypeptides. This synthesis can be induced particularly by a septic wound or, generally speaking, trauma or by immunization, that is to say, by injection of a low dose of bacteria.

Among the peptides or polypeptides that are produced, we can distinguish the following four groups:

(i) the cecropines, which are cationic peptides of 4 kDa forming two α-amphipathic helixes; (ii) cationic peptides that are rich in proline, rather small in size (2 to 4 kDa), partly characterized as the apidaecines and the abaecines; (iii) several distinct polypeptides having a molecular weight (PM) of 8 to 27 kDa, mostly cationic and frequently rich in glycine residues such

[1] Numbers in the margin indicate pagination in the foreign text.

as the attacines, sarcotoxins II, the diptericines and the coleoptericines, and (iv) the defensines, nonglycosylated peptides, moderately cationic with a pI of 8.0 to 8.5, comprising 38 to 43 aminated acids and containing a characteristic charge of 6 cysteine residues inserted in 3 intramolecular disulfide ridges.

The antibacterial peptides mentioned here were induced among the lepidoptera, diptera, hymenoptera and coleoptera. Work done so far on other species did not make it possible, on the other hand, to detect their presence. /2

It should be noted that as of this day the chemical structure attributed to these peptides would correspond to a simple chain of aminated acids.

On the basis of this discovery, the expert in the field, seeking by way of synthesis to prepare peptides having such properties, would thus be inclined only to construct a peptidic sequence by chain-linking aminated acids determined according to conventional techniques.

Now in the context of their work on the study of the molecular bases of the immunity response of insects, the inventors found that the antibacterial activity of certain peptides that can be induced among particular species of insects was tied to the presence of certain substitution groups in aminated acids.

The elimination of these groups leads to a very considerable reduction in antibacterial activity, even its complete disappearance.

The object of the invention therefore is to provide new substituted peptides that have a high activity level with respect to a large number of bacteria.

It is furthermore intended to supply processes for the procurement of such peptides that will make it possible to obtain them in large quantities.

The invention furthermore is intended to make use of the biological properties conferred by the groups of substitutions in preparing antibacterial agents that can be used in human and veterinary therapy and, generally speaking, for the fight against bacteria.

The peptides according to the invention are characterized in that they have antibacterial properties conferred by a sequence of aminated acids enclosing at least one aminated -O-substituted acid by a glycosyl group containing one or several ose and/or osamine charges, a poly-ose and/or poly-osamine.

The substituted aminated acid is a hydroxylated aminated acid such as threonine, serine or tyrosine. Threonine is particularly preferred. /3

The substitution group of the aminated acid or acids will be designated in the following description and in the claims by the

term "glycosyl" and the peptides of the invention are also called glycopeptides.

The glycosyl group is linear or cyclic, substituted or not, in the form of α or β. It advantageously comprises charges of ose and/or osamine of 5 or 6 carbon atoms.

As appropriate ose groups, we might mention the pentoses such as ribose, lyxose, xylose, arabinose and hexoses such as galactose and glucose, fucose, tallose, altrose, gulose, mannose and allose.

The osamine groups are advantageously made up of a galactosamine, glucosamine, tallosamine, altrosamine, gulosamine, mannosamine or allosamine radical.

The poly-oses and/or poly-osamines comprise a series of ose and/or osamine charges, in particular, 2 to 10 charges, especially 2 to 7 charges.

According to a variant of the invention, the glycosyl group is substituted.

The substituents of the ose and/or osamine charges are then advantageously chosen from among the alkyl radicals with 1 to 4 carbon atoms or, in particular, in the case of the osamines, among the acyl radicals, more especially acetyl, said radicals more particularly substituting the amine function.

These substituents can also correspond to at least one ose and/or at least one osamine charge. The glycosyl group can thus

comprise an osamine charge bearing one or several alkyl and/or acyl groups and it can also be substituted by one or several ose charges, in particular, 2 or 3.

As an example of a substituted glycosyl group, we might mention a glucosamine or galactosamine charge, substituted by one or several glucose and/or galactose charges, in particular, an N-acyl-glucosamine or N-acylgalactosamine charge, substituted by one or several glucose and/or galactose charges, in particular, 2 or 3. /4

Other glycopeptides of the invention comprise more complex substitutions involving the terminal N and/or C parts and/or substitutions by radicals of sialic acid.

As shown by the work of the inventors reported in the examples, the glycosylic groups mentioned rather astonishingly appear indispensable in having an appreciable level of antibacterial activity.

The glycopeptides according to the invention furthermore are characterized in that they are of the type of glycopeptides as obtained among the arthropods and, in particular, from the larvae or the adults of insects, by a process comprising the following:

- the induction of their synthesis, especially by injection into insects of bacterial in sufficient doses, or by septic wound or other trauma,

- extraction so as to collect the peptides that underwent post-translational glycosylation and, as the case may be,

- the fractionation of the isolated extract so as selectively to separate the glycopeptides according to the level of their antibacterial activity.

According to a variant, the extraction is performed on the whole animal, which beforehand has been frozen and then ground up.

According to another variant, extraction is performed on the hemolymph of the animals; this is done in the conventional fashion so as to separate the basic peptides.

The invention involves corresponding extracts, mixtures of glycopeptides and fractions.

The glycopeptides of the invention are also characterized in that they present an antibacterial activity against gram-negative and gram-positive germs.

A group of glycopeptides such as those defined above is also characterized in that their peptidic sequence responsible for the antibacterial activity is contained in a domain comprising at least 30% of proline groups approximately. /5

This chain-link setup more especially comprises a consensus charge of O-glycosylation proline-threonine/serine - Xaa1-Xaa2-proline in which the Xaa1 and Xaa2 charges, identical or different, are variable aminated acids.

The chain-link setup of aminated acids in the biologically active sequence, that is to say, presenting an antibacterial activity, advantageously comprises at least one hydrophobic aminated acid such as threonine substituted by a glycosyl group.

The glycosyl substitution group is more particularly made up of an N-acylhexosamine charge, more especially N-acetylgalactosamine or N-acetylglucosamine, which, as the case may be, is itself substituted by one or several oses, especially pentoses and/or hexoses such as fucose and/or galactose and/or glucose.

Preferred glycopeptides are those that can be induced among the brachycera.

One group of these glycopeptides, which is advantageous with respect to its antibacterial properties, is a group that can be induced among drosophila.

The chain-link setup of aminated acids of glycopeptides of this group is, in particular, comprised of or constituted of the following (I) sequence as determined according to the graduation of Edman:

this sequence and the following sequences are shown in the part entitled "List of Sequences" given at the end of the specifications with the identifications SEQ ID No. 1 and thereafter.

[Please insert sequence on original page 5].

[Please insert sequence, top, original page 6]. /6

More particularly, said chain-link setup is made up of or comprised in the following sequence (II) (SEQ ID No. 2) of aminated acids.

[Please insert sequence, center, original page 6].

This sequence corresponds to the chain-link setup extending from positions 22 to 40 in sequence (I).

In these preferred glycopeptides corresponding to these sequences, the threonine or serine charges are substituted by a glycosyl group. In particular, one might mention the glycopeptides in which the threonine charge is substituted by an N-acylhexosamine group such as N-acetylglucosamine or N-acetylgalactosamine, said group itself comprising one or several oses such as fucose, glucose or galactose.

Other preferred glycopeptides of the invention are of the type of those that can be induced among the diptera and, among the latter, especially among the Phormia terranovae.

Such glycopeptides advantageously comprise or are made up of a chain-link setup of aminated acids as determined according to the graduation of Edman, responding to the following sequence (III) (SEQ ID No. 3):

[Please insert sequence, bottom, original page 6].

[Please insert sequence, top, original page 7]. /7

in particular, in the following sequence (IV) (SEQ ID No. 4):

[Please insert sequence, bottom, original page 7].

Other chain-link setups comprise as the N-terminal part at least one part of the following sequence (V) (SEQ ID No. 5):

[Please insert sequence, top, original page 8]. /8

in particular, in the following sequence (VI) (SEQ ID No. 6):

[Please insert second sequence on original page 8].

or the following sequence (VII) (SEQ ID No. 7):

[Please insert third sequence on original page 8].

Other sequences of aminated acids of peptides endowed with antibacterial properties such as they are inducible in Phormia terranovae respond to the following sequence VIII (SEQ ID No. 8):

[Please insert sequence, bottom, original page 8].

[Please insert sequence, top, original page 9]. /9

In sequences (III) to (VIII), threonine or serine in position 10 and/or the one in position 54 is (are) substituted by a glycosyl group as defined above.

The glycosyl group is advantageously an osamine charge, which itself is substituted by one or several ose charges. A glycosyl group suitable for arranging antibacterial glycopeptides corresponds to an (N-acylhexosamine)-hexose group, in particular, N-acetyl-galactosamine or N-acetyl-glucosamine; this group is itself substituted by one or several ose charges, in particular, fucose and/or galactose and/or glucose.

Other preferred glycopeptides are those that can be induced among the hymenoptera, especially among the bees.

Still other glycopeptides are of the type of those that can be induced among the hemiptera, for example, Pyrrhocoris apterus.

Particularly preferred glycopeptides of this type comprise or are made up of a chain-link setup of aminated acids as determined according to the graduation of Edman, presenting the following sequence (IX) (SEQ ID No. 9):
[Please insert sequence, bottom, original page 9].

[Please insert sequence, top, original page 10]. /10

In this sequence, the threonine charge is in position 11 and is substituted by a glycosyl group as defined above.

The invention also relates to fragments or variants of glycopeptides defined above; hence these fragments or variants are recognized by antibodies that are directed against said peptides and/or possess characteristics of charge and/or hydrophobicity or hydrophilia, conferring upon them an antibacterial activity against gram-negative germs as observed in the native glycopeptides. By variants, we mean here glycopeptides in which one or several aminated acids are deleted, substituted by a group other than a glycosyl or replaced by another aminated acid having a neighboring charge.

The characteristics of hydrophilia and hydrophobicity of aminated acids are given by Kyte et al. in J. Mol. Biol., 157, 105-132, 1982.

Antibodies formed against glycopeptides and their fragments and variants also are covered by the scope of this invention.

According to another aspect, the invention contemplates sequences of nucleotides that contain genetic information corresponding to the aminated acids of the glycopeptides defined above.

It also covers sequences of nucleotides capable of being hybridized under stringent conditions with the above sequences.

By stringent conditions, we mean the conditions defined in the work "Molecular Cloning" by Sambrook, Fritsch, Maniatis, 1989, Cold Spring Harbor Laboratory Press.

The invention furthermore relates to sequences of nucleotides that are complementary to those defined above such as the corresponding ARN.

The carriers for expression and/or cloning, comprising at least one fragment of sequences of nucleotides defined above, are also part of the invention, as are the hosts that are transformed by these carriers. /11

By way of example, hosts suitable for the implementation of the invention comprise bacteria such as E. coli, yeasts or also cells of arthropods, vertebrates or plants.

The invention also relates to products for the expression of the above carriers.

The chemical structure of glycopeptides with antibacterial activity has been characterized as indicated above so that the expert in the field will easily select the techniques that are most appropriate for their preparation.

Working via synthesis, one easily gains access to structures of glycopeptides such as those that can be induced among the arthropods or their variants.

Advantageously, one substitutes one or several of the aminated acids of the sequence with a glycosyl group, then one introduces groups that will block the functions to be protected and one forms a peptidic chain, advantageously determined with the help of a synthesizer, while the aminated acid or acids are sequentially introduced so as to occupy the desired position in the sequence. The blocking groups are eliminated at the end of synthesis.

As a variant, one prepares the peptide with a particular protection for the aminated acid to be substituted by the glycosyl group.

One then proceeds to the elimination of the group that protects this aminated acid only, after which one introduces the protected glycosyl group. The groups that protect other aminated acids and those of glycosyl are then eliminated.

According to a variant, these glycopeptides can be obtained according to the techniques of genetic engineering by introducing

in an appropriate carrier the sequences of nucleotides that are capable of expressing the peptidic skeleton of the desired glycopeptide and by making the expression in a host capable of ensuring post-translational glycosylation. As a variant, when the host used cannot ensure this functionalization, one resorts to the ways of chemical synthesis. /12

According to yet another variant, these glycopeptides are obtained by immunizing arthropods, including genes capable of coding for the desired glycopeptide(s), especially through injection of bacteria in a sufficient quantity to cause their synthesis or by traumatism such as a septic wound. Several hours after immunization or after the trauma, the glycopeptides are extracted and then fractionated and the fraction or fractions corresponding to the desired glycopeptide(s) are isolated.

Extraction is performed under conditions that make it possible to prevent any phenomenon of degradation of glycopeptides such as oxidation or the action of proteases.

For purification of products, one advantageously uses very little retentive supports that also have a high separating power. Appropriate supports are chosen from among the supports used in the inverse phase.

The implementation of these arrangements makes it possible to isolate the glycopeptides in a pure form from animals as small as the adults of drosophila.

In particular, we note that this extraction technique, as described in the examples, makes it possible to isolate glycopeptides whose fragility is well known.

The antibacterial properties conferred upon the peptides of the invention by the glycosyl group were evidenced through tests on the inhibition of the growth of gram-negative bacteria and gram-positive bacteria. Results are given in the examples by way of illustration. Pathogenic bacterial cultures such as the enterobacteria performed on the basis of samplings on patients were then placed in an agar-containing box. The deposit of glycopeptides according to the invention on these bacteria showed their great bactericidal effectiveness. These properties are used for preparing antibacterial agents that can be used in human or animal therapy or to treat a given environment. /13

The invention thus contemplates the use for the purpose of preparing antibacterial agents and compounds of peptides that contain at least one aminated -O-substituted acid by group containing one or several oses and/or osamine charges, one poly-ose and/or poly-osamine possessing an antibacterial activity conferred by the glycosyl substitution group(s).

These compounds are characterized in that they encompass an effective quantity of glycopeptides defined above in association with an inert vehicle that is appropriate for the contemplated practical use.

It is advantageous to incorporate excipients such as the protease inhibitors, antioxidants and inhibitors for the multiplication of bacteria.

Compounds or agents intended for human or veterinary therapy are presented advantageously in the form of injectable solutions or in a form for external use such as ointments, creams, powders or solutions.

They are particularly indicated in case of septicemias or furthermore in the treatment of eyes, ears, care of the mouth and the teeth and in gynecology.

Advantageously, one resorts to the posologies that are customary in these fields.

The compounds of the invention also are very worthwhile in agricultural chemistry as plant health agents. They are advantageously used in the form of powders or solutions for spreading.

The use of the antibacterial compounds and agents of the invention in the agribusiness industry makes it possible to prevent contamination by gram-negative germs during the manufacture of products and their conservation.

The invention also covers the genomes of plants such as those that are modified by the presence of genes that code for the above-defined antibacterial glycopeptides. these vegetable and plant cells are chosen from among that are capable of

ensuring the glycosylation of the peptidic sequences so as to confer an antibacterial activity upon them. We thus get plants that will resist pathogenic agents. /14

By way of illustration, the following examples will offer some other characteristics and advantages of the invention. In these examples, reference is made to Figures 1 and 2, which respectively show the following:

- Figure 1 shows a variation of absorbance as a function of the time of a peptide eluted according to the invention and

- Figure 2 shows sequences of nucleotides capable of coding for a peptide of the invention.

Example 1: Obtaining antibacterial glycopeptides by extraction from drosophila.

- Biological synthesis induction method.

The drosophila are anesthetized by means of carbon dioxide and are immunized by puncture on the thoracic level in the proximity of the wing insertion with the help of a needle that is stuck before each inoculation into a bacterial suspension of Micrococcus luteus (gram-positive) and of Escherichia coli 1106 (gram-negative). The insects are kept at 25°C for 24 hours and are killed by freezing in liquid nitrogen.

- Extraction, fractionation and purification of synthesized glycopeptides:

* Extraction

The legs, wings and heads of the adults of drosophila are removed by screening, the chests and the abdomens are ground in the presence of liquid nitrogen for 30 minutes in a mortar kept cold. To the powder thus obtained, one adds 10 volumes of acidified water (0.1% of trifluoroacetic acid (TFA 0.1%)) containing aprotinine (2 mg total). After 30 minutes of extraction while stirring, the extract is centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernatant thus obtained is immediately subjected to the different stages of purification./15

* Fractionation of the extract on Sep-Pak C18 cartridges

After the extract has been deposited on an inverse-phase support as sold in the form of cartridges under the brand name Sep-Pak C18[B] by Waters Millipore, the molecules with a hydrophile character are eliminated by simple washing with 5 ml of acidified water (TFA 0.05%). The elution of hydrophobic molecules is performed with solutions of 10, 40 and 80% acetonitrile in acidified water (TFA 0.05%).

The fractions thus collected are called "Elution 10%," "Elution 40%" and "Elution 80%" and are concentrated in a vacuum. The fractions are then reconstituted with Milli Q water prior to HPLC analysis.

* Purification by means of HPLC of molecules displaying antibacterial activity.

a - First purification stage

The "Elution 10%" fraction is analyzed on an inverse phase Aquapore OD 300 C18® column with a linear gradient of acetonitrile from 0 to 40% in acidified water (TFA 0.05%) over a period of 90 minutes (in other words, an increase of 0.44% of acetonitrile per minute) for a flow rate of 1 ml/min.

The "Elution 40%" fraction is analyzed on the same fraction as the "Elution 10%" fraction. Elution is performed in a linear gradient of acetonitrile from 2 to 52% in acidified water (TFA 0.05%) over a period of 90 minutes (increase of 0.55% of acetonitrile per minute) at a flow rate of 1 ml/min.

The "Elution 80%" fraction is analyzed on an inverse-phase Aquapore RP 300 C8 column. Elution is performed in a linear gradient of acetonitrile from 10 to 60% in acidified water (TFA 0.05%) over a period of 90 minutes (in other words, an increase of 0.055% of acetonitrile per minute) for a flow rate of 1 ml/min.

The fractions are collected according to the variation of absorption at 225 nm, taking into account the shoulders. Each fraction thus collected can be attributed to an optical density peak.

/16

All fractions are dry-evaporated under a vacuum and are reconstituted in Milli Q water. The antibacterial activity of each fraction is detected by the technique of the growth

inhibition test in a liquid medium on aliquot fractions of 10 µl: one such fraction is equivalent to an extract of 300 adults of drosophila.

b - Final purification

Two supplementary stages are necessary for the final purification of molecules with antibacterial activity. The "Elution 10%" fractions that present biological activity are analyzed on an inverse-phase Aquapore OD 300 C18® column. Elution is performed in an acetonitrile linear gradient of 0 to 20% in acidified water (TFA 0.05%) over a period of 90 minutes (in other words, an increase of 0.22% of acetonitrile per minute) for a flow rate of 0.8 ml/min.

The fractions that present antibacterial activity, measured according to the method indicated below, are purified on the previously described column in a linear gradient of acetonitrile of 0 to 20% in acidified water (TFA 0.05%) over a period of 90 minutes for a flow rate of 2 ml/min.

In Figure 1, we show the variation of the optical density DO at 225 nm as a function of the time during the stage of final purification of glycopeptide. The dotted line shows the acetonitrile (in terms of percent) added for purification. The solid-line arrow (↓) designates the purified glycopeptide and the one in dots ( ) designates the synthesis peptide that will be

discussed later. The column (‖) represents the antibacterial activity.

The molar mass measured is 2564.4 daltons.

The analysis of the microsequence is performed by proceeding to an alternated Edman graduation of the peptide and the detection of the derivatives of phenylthiohydantoin on an automatic pulsed liquid sequencer (Applied Biosystems) model 473 A or 477 A. /17

The analysis of the aminated acids is performed on a 420 A (Applied Biosystems) analyzer with HPLC analyzer and microcoordinator.

The peptide is hydrolyzed at 120°C for 24 hours with HCl 6N in the gaseous phase (Pico-Tag, Millipore). One forms derivatives with phenyl isothiocyanate and then one detects the aminated acids at 254 nm.

The isolated and purified peptide corresponds to the sequence (II) in terms of aminated acids given earlier and comprises a substitution group N-acetylgalactosamine-galactose on the threonine charge as demonstrated by HPLC and mass spectrometry.

The biological activity was measured by the following method:

* Preparation of strains for the antibacterial test

For each strain, an isolated colony is removed and suspended in 30 ml of PB medium (LB medium devoid of yeast extract) and it is incubated at 30°C for one night while stirring slowly. The bacteria in the exponential growth phase are restored by dilution in fresh PB medium at a concentration of 400,000 UFC (colony-forming units)/ml for E. coli and 40,000 UFC/ml for M. luteus (in other words, DO_{600} = 0.001).

* Performance of antibacterial test.

The sample to be tested (10 μl) is placed in wells of microtitration plates to which are added 100 μl of a bacterial culture in the exponential growth phase brought to DO_{600} = 0.002. After 24 hours of incubation at 25°C, the bacterial growth is measured at 600 nm. The bacteria growth inhibition, which reflects the antibacterial activity, is evidenced by the difference of DO_{600} existing between the tested fractions and a control culture in which the 10 μl of sample are replaced by 10 μl of sterile water.

The results obtained are given in the table below and are expressed in terms of UFC/ml x 10^3. /18

Incubation time	1 min	1 hr	3 hrs	5 hrs	24 hrs
Antibacterial glycopeptide	1088	1026	688	600	323
Control experiment	1220	1164	1200	1250	1088

These results clearly point up the bactericidal activity of the products involved in the invention.

For comparison, we prepared -- via synthesis using a peptide synthesizer just as the one indicated above -- a peptide presenting the aminated acid skeleton of chain-link setup I but in which the threonine in position 11, contrary to the glycopeptide of the invention, is not glycosylated.

The sequence of synthetic peptide was checked by performing an Edman graduation and by determining the molar mass with the help of a VG Biotech Bio Q mass spectrometer.

We find a different elution of peptide in HPLC, as shown in Figure 1.

The determination of the molar mass yields a value of 2199.6 daltons, which differs by 364.8 daltons from that of the glycopeptide given in Example 1.

Moreover, this synthetic peptide, devoid of the glycosyl grouping, presents a biological activity that is 10,000 times less than that of the glycopeptide in Example 1.

To prove that the difference between the glycopeptide and this synthetic peptide comes from the O-glycosylation on threonine in position 11, the purified glycopeptide in Example 1 was subjected to the action of anhydrous HF, which specifically hydrolyzes the O-glycosyl bonds.

This treatment primarily yielded a compound of 2199.6 daltons, hence having the same mass as the unsubstituted synthetic peptide and a compound of 2403.0 daltons. On the other hand, the same treatment, applied to synthetic peptide, does not alter the latter whose molar mass remains unchanged. /19

The glycosyl group is identified as being an N-acetylgalactosamine-galactose group.

Example 2: Sequences of nucleotides coding for antibacterial glycopeptides.

. Isolation of clones of ADNc:

With the help of a degenerated probe 5' TAG GGN CGN GGC TTG CC 3' and starting with 30,000 phages containing the corresponding insert, one proceeds to the screening of a bank of ADNc of drosophila (the preparation of this bank is described below).

One gets 30 positive hybridation clones.

. Sequencing

Ten of these clones are sequenced. One finds that all of the inserts contain an open reading frame of 65 codons, commencing with a methionine. Inserts 1 to 9 do not show any sequence variation. The longest one is shown in Figure 2 (SEQ ID No. 10). Insert 10 (SEQ ID No. 11) shows variations of bases that are represented in Figure 2. The consensus signal of polyadenylation AATAAA is indicated in bold characters. The

first line of the sequence of nucleotides corresponds to those of clones 1 to 9, the second line mentions the different bases of clone 10, the other bases being identical to those given on the first line are not indicated.

The deduced sequence of aminated acids corresponds to a precursor of the peptidic sequence of the glycopeptide given in Example 1 and presents the chain-link setup I given above. This chain-link setup I comprises sequence II of 19 aminated acids and in the N-terminal part a potential peptidic signal (charges 1 to 19) and a Thr-Pro dipeptide (in positions 20 and 21). The C-terminal part comprises a peptide of 22 aminated acids (occupying positions 43 to 64 in chain-link setup I), separated from chain-link setup II by a dibasic Arg-Arg cleavage (in positions 41 and 42). /20

. Preparation of ADNc bank

One prepares an ADNc bank of the size selected by submitting to a bacterial challenge some larvae of drosophila in the third larval stage. The ARN, enriched with poly (A), is extracted as described by Reichhart et al. in EMBO J. 11, 1469-1477 (1992). A λ gt 22 bank is constructed by using the Superscript lambda system sold by Gibco BRL. The ADNc are sub-cloned in Mt3mp19. The two young seedlings are sequenced using the method of Sanger with dideoxy, employing the sequencing kit sold under the brand name Sequanase® by USB.

Example 3: Procurement of antibacterial glycopeptides by expression in Saccharomices cerevisae.

Using the conventional techniques of genetic engineering, one inserts in a carrier a sequence of nucleotides of a clone of ADNc from Example 2 and one makes the expression in Saccharomices cerevisae.

The glycopeptide thus produced is recovered and analyzed to check its sequence.

Example 4: Procurement by way of synthesis of antibacterial glycopeptides of the type of those induced in Phormia terranovae.

One produces a reaction between threonine and an N-acetylgalactosamine. One blocks the hydroxyl groups of galactosamine with the help of protector groups that are customarily employed for this purpose. One uses, for example, benzoyl chloride to protect the hydroxyl groups with a benzoyl radical.

The chain-link setup of aminated acids of sequence VI, given earlier, is put together with the help of a peptide synthesizer as used in Example 1.

The mass spectrometry analysis of the product thus obtained and the graduation of Edman make it possible to check that the desired glycopeptide was properly synthesized.

Example 5: Procurement of antibacterial glycopeptides by extraction on the basis of Phormia terranovae. /21

- Induction of biological synthesis.

Larvae of the third stage of Phormia terranovae are wounded by puncture with the help of a needle that is beforehand stuck into a bacterial suspension, for example, of Enterobacter-cloacae.

- Extraction, fractionation and purification of synthesized glycopeptides.

After 24 hours, the blood is sampled by incision of the cuticle and is subjected to centrifugation at 36,000 g for 20 minutes at 40°C.

Acetic acid is added to the supernatant and the latter is heated to 100°C for 4 minutes. The precipitated proteins are eliminated by centrifugation and the supernatant is applied upon a cation exchange column, balanced with a 40 to 500 mM buffer of ammonium acetate.

One checks the biological activity of the peptides thus obtained by proceeding as described in Example 1, for example, by studying the activity with respect to E. coli. The active peptides thus obtained are filtered on Sep Pack C18 Waters® cartridge and are purified via liquid chromatography, inverse-phase HPLC on a Baker-Bond C19 WP® column. Elution is performed according to a linear gradient with acetonitrile.

One subjects one of the freshly isolated active peptides to the Edman graduation and one determines its molecular mass.

The sequence of aminated acids, thus obtained, responds to sequence (VIII) given earlier; its molecular mass is 9440.4 (± 3). The threonine residues in positions 10 and 54 are O-glycosylated. This O-glycosylation corresponds on each of these positions to an N-acetyl hexosamine-hexose group. In particular, the threonine residues are substituted by N-acetylgalactosamine-galactose-glucose groups. By treatment with HFA hydrofluoric acid, one observes a diminution in the molecular mass to 8692.6 and a loss of biological activity.

As in the case of the peptide of Example 1, we will be interested to note that O-glycosylation is necessary for the biological activity of the peptides. /22

These substitutions can be more complex than those already indicated, corresponding to an N-acetyl-hexamine-hexose group. As a matter of fact, the extractions of active peptides were performed in an acid medium and can thus lead to an alteration of the substitution chain.

Working under gentler conditions, it is thus possible to obtain active peptides comprising a glycosyl group that is furthermore substituted, for example, by a radical of sialic acid and/or a fucose.

Example 6: Procurement of antibacterial glycopeptides from Pyrrhocoris apterus.

- Immunization and procurement of hemolymph.

One collects the adults of P. apterus in the summer on leaves of hibiscus. One injects 1,000 insects with a suspension of 2 μl containing 2,500 cells of M. luteus (gram-positive bacteria) and 2,500 cells of E. coli (gram-negative bacteria).

At different time intervals, one collects the hemolymph (about 3 μl/insect), sectioning an antenna and gently pressing the body of the insect. The hemolymphs are assembled and placed in a well of precooled plastic in the presence of aprotinine (Sigma A-62-79, final concentration: 10 μl/ml of hemolymph) as protease inhibitor and phenylthiourea (final concentration: 1 μg/ml of hemolymph) as melanization inhibitor. The hemolymph is centrifuged at 13,000 g for 1 hour at 4°C.

One observes an intensive antibacterial activity during the period of 24 hours following the injection. This activity is maintained up to 72 hours. The total volume of hemolymph collected is 2.2 ml.

After elimination of the blood cells by centrifugation, one applies the supernatants, as indicated in the examples above, upon a Sep-Pak C18® cartridge.

Antibacterial activity is recovered by elution with 50% acetonitrile in acidified water (0.05% TFA). /23

The active fractions are collected and applied on a cut exclusion HPLC column; then one performs isocratic elution with 30% acetonitrile in acidified water (0.03% TFA).

The antibacterial activities of the fractions are checked by dosing aliquot quantities and by preforming growth inhibition tests on plates with respect to different microorganisms. An anti-E. coli activity is detected in fraction 1, corresponding to molecular masses of 10 to 30 kDa, and an antibacterial activity directed against E. coli and M. luteus in fraction 2 with molecular masses of less than 10 kDa.

Fraction 1 is then subjected to HPLC C18 in the inverse phase and the active molecules are first of all eluted with a gradient varying from 2 to 52% of acetonitrile in acidified water (90 minutes, flow rate 1 ml/min). One performs a new chromatography on the same column with a lesser gradient of 15 to 35% acetonitrile in acidified water (for 90 minutes) with a greater flow rate (2 ml/min). One gets a product that appears to be pure; it is called peptide A.

Fraction 2 is also subjected to HPLC C18 chromatography in the inverse phase with a gradient of 2 to 25% of acetonitrile in acidified water (0.05% TFA).

Two zones displaying an antibacterial activity are recovered: one corresponds to a peptide called peptide B, presenting an anti-M. luteus activity, and the second one corresponds to a peptide called peptide C, which is active with respect to E. coli.

Peptide C appears to be constituted by a mixture of compounds and is subjected to supplementary purification with the help of two successive processes of chromatography (HPLC, gradient of 10 to 30%, followed by a gradient of 5 to 25% of acetonitrile in acidified water, 0.05% of TFA, 90 minutes for each gradient). One thus gets peptide C in the pure form. /24

- Determination of the primary structure of peptide C.

One subjects peptide C to the Edman graduation reaction.

The determination of the sequence of aminated acids shows that peptide C comprises 20 residues and corresponds to chain-link setup IX given above. Measurement of the molecular mass by means of mass spectrometry yields a value of m/z = 2543.3.

The signals observed for the residue in position 11 are similar to those observed for the threonine residue of peptide according to Example 1 as induced in drosophila.

The difference between the calculated mass of peptide (2340.2 Da) and the experimental mass (2543.3 Da) is 203.1 Da; this corresponds to an N-acetylhexosamine (221.18 Da) charge with one molecule of water necessary for the glycan link. The threonine charge substitution group is an N-acetylgalactosamine group.

Example 7: Formulary that can be used to treat septicemias with enterobacteria.

1) Peptide from Example 1: 10 to 100 mg